KARNATAKA STATE AKKAMAHADEVI WOMEN'S UNIVERSITY, VIJAYAPURA
Jnanashakti Campus, Torvi, Vijayapura

Title of the research work:

''Investigation on antihyperlipidemic effects of minor millets and their malt proteins through transciptomic analysis''

Pre-Synopsis

Submitted towards the partial fulfillment of Ph.D programme in Bioinformatics

Submitted By

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Under the Guidance of

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From:
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Date: 05/03/2019

To,
The Deputy Registrar (Academic)
Karnataka State Akkamahadevi Women's University,
Vijayapura

Through Proper Channel

Respected Sir,

Sub: Submission of pre-synopsis - regarding

Ref: KSWU/Acad-03/Ph.D/2017-18/3114.

I am here with submitting 3 copies of pre-synopsis for my Ph.D. work. This is for your kind information and perusal.

Thanking you,

Place: Vijayapura

Yours faithfully
(Nivedita Pujari)

Enclosures:

1. Ph.D study Progress report-1.
2. Syndicate Bank Challan copy.
3. Copy of Ph.D admission letter.

Copy to,

1. Chairman- Dept. of Bioinformatics, KSAWU Vijayapura
2. Dept. of Bioinformatics, KSAWU Vijayapura.
3. Prof C K Ramesh Former, professor Department of bioinformatics, KSAWU Vijayapur. Currently, professor Department of Biotechnology, Sahyadri Science College, Shivamogga

‘‘Investigation on antihyperlipidemic effects of minor millets and their malt proteins through transciptomic analysis’’

INTRODUCTION

Hyperlipidemia, a pathophysiological condition with elevated levels of lipids in the blood projects injurious effects on functionality of vital organs including heart, liver and kidney. Thus management of this condition is global health related issues. Increase in population, economic prosperity, urbanization and aging has lead to rapid transition in epidemiology of hyperlipidemia and its associated risk factors. Increase in risk of hyperlipidemia is mainly associated with adverse lifestyle such as alteration in nutritional intake with greater amount of unhealthy diets, smoking, tobacco use etc. (Gupta and Gupta, 2009). Hyperlipidemia resembles the increase in any or all of the lipids (cholesterol, triglycerides, phospholipids, and fatty acids). Therefore elevated levels of these lipid levels are reported to cause hyperlipideamic condition and also lead to several other disorders associated with hyperlipidemia. It involves the loss of balance between low density lipoprotein cholesterol and high density lipoprotein cholesterol in blood. This imbalance may further increase risk of cardiovascular diseases, obesity, diabetes, cancer and inflammatory conditions (Karr and Samantha, 2017).

Little millet

porso millet

foxtail millet

Barnyard millet

kodo millet

The pharmacological interventions in managing the hyperlipidemia conditions are associated with prominent and life threatening side effects and thus limiting their applications. Diet is considered to be an appropriate alternative in managing or treating many of the diseases and therefore the proposed study is focused on development of diet based therapy against hyperlipidemia condition using millets. Millets are cereal crops belong to one of the most ancient food grains and are grown in different parts of Asian countries since 2700 BC. They are known to be originated in eastern Asia, but however one third of the world's population uses them regularly as there every day food. Millets are produced in the highest rate by India and are known to be staple food for several lives from rich to poor (Gupta et al., 2010). According to Indian Institute of millets, they are classified into major and minor or small millets. Small millets consist of six different species which includes finger millet, little millet, foxtail millet, barnyard millet, porso millet, and kodo millet. While major millets are sorgham and pearl millet. Minor millets or small millets are known to be the less utilized cereal crops which are capable of growing in different climatic conditions (Desai et al., 2010). Minor millets or small millets are abundant in vitamins, minerals, micronutrients such as iron, calcium, and zinc. They are also rich source of energy, dietary fiber, phytochemicals with antioxidant properties (Bouis, 2000) and thus are considered to be nutri-cereals. There are reported to have several compounds that help against chronic diseases associated with hyperlipidemia like ischemic stroke, cardiovascular diseases, cancer, obesity and diabetes. (Jones et al., 2000, Jones, 2006). There are several reports that provide evidences for treating hyperlipidemia through millet based diets (Kumara and Thyumanavan, 1997; Park et al., 2008; Lee et al., 2010). With this background we shall evaluate the antihyperlipidemia potential of millet and develop millet based product for management of hyperlipidemia.

OBJECTIVES:

1. Investigations on antihyperlipidemia property of 5 different types of minor millets.
2. Unmasking the molecular mechanism of antihyperlipidemia action of potential millet through transcriptomics analysis.
3. Analysis of gene expression in response to therapeutic action of millet against hyperlipidameia through bioinformatics approach.

REVIEW OF LITERATURE

Several treatments are reported to manage hyperlipidemic conditions. National cholesterol education program was established by national institute of health in 1987 have proposed the management of hyperlipidemia through the use of Stains, Ezetimibe, Bile acid sequestrants, Niacin, Fibrates, and plant sterols (Hemphill, 2010). However, complementary and diet based medicines when compared to pharmaceutical products are better and safer alternatives which includes nuts, plant stanolols, psyllium, soluble fiber, orange juice, and red yeast rice (Nies et al., 2006).

Although statin based therapy by using atorvastatin, rosuvastatin, fluvastatin, and pitavastatin has made considerable amount of progress in their usage in treating hyperlipidemia, there are several side effects reported that might pose chronic health issues. Even though statins have been confirmed to show safety and efficient profile, they have reportedly showed evidences of causing several adverse side effects, and the condition is known as statin intolerance (Weng et al ., 2010). Statins are known to negatively affect fatty acid oxidation, sarcolemal cholesterol, deplete isoprenoid, disturb calcium homeostasis and lipid metabolism, which cause autoimmunity, and increase cellular lipids leading to emergence of statin-induced adverse effects (Stone, 2004). Reports also indicate that continuous usage of statins for longer period of time was noticed to increase the risk of diabetes (Sattar et al., 2011). Another study also reported that hyperlipidemic patients treated with intensive stain therapy showed faster risk of developing diabetes then compared the ones treated with moderate statin therapy (Preiss et al., 2010). There are evidences that have reported to cause liver dysfunction by continuous usage of statin therapy eventually leading to liver failure (Russo et al., 2009).

Millets are cereal crops belong to one of the most ancient food grains and are grown in different parts of Asian countries since 2700 BC. They are known to be originated in eastern Asia, but however one third of the world's population uses them regularly as there every day food. Millets are produced in the highest rate by India and are known to be staple food for several lives from rich to poor (Gupta et al., 2010). According to Indian Institute of millets, they are classified into major and minor or small millets. Small millets consist of six different species which includes finger millet, little millet, foxtail millet, barnyard millet, porso millet, and kodo millet. While major millets are sorgham and pearl millet.

Minor millets or small millets are known to be the less utilized cereal crops which are capable of growing in different climatic conditions (Desai et al., 2010). Minor millets or small millets are abundant in vitamins, minerals, micronutrients such as iron, calcium, and zinc. They are also rich source of energy, dietary fiber, phytochemicals with antioxidant properties (Bouis, 2000) and thus are considered to be nutri-cereals. They are reported to have several compounds that help against chronic diseases associated with hyperlipidemia like ischemic stroke, cardiovascular diseases, cancer, obesity and diabetes. (Jones et al., 2000, Jones, 2006). There are several reports that provide evidences for treating hyperlipidemia through millet based diets.

There are several evidences that are reported in Indian population on consumption of millets showing association with decrease lipid levels. Kumara and Thyumanavan, (1997) reported starch from barnyard millet based treatment to rats produced lowest blood glucose, serum cholesterol, and triglycerides levels in blood than compared with rats fed with rice. Another study showed that genetically obese mice with type 2 diabetic under high fat feeding conditions when fed with porso millet protein showed improved plasma levels of high density lipoprotein (HDL) (Park et al., 2008). Lee et al., (2010) reported that finger millet and porso millet show reduction in plasma triglycerides in hyperlipidemic rats. Germinated finger millets were used for solid state fermentation of *Monascus purpureus* (Venkateswaran and Vijayalakshmi, 2010) which showed antihypercholermic metabolites. Other studies on

treatment using phenolic extracts from kodo, finger, proso, foxtail, little, and pearl millets showed inhibitory effects on lipid peroxidation (Chandrasekara and Shahidi, 2011).

According to disease control data survey, hyperlipidemia is ranked second in the 10 most chronic diseases in India (year). Hyperlipidemia is proved to strongly associate with many chronic diseases such as diabetes, obesity, cancer, and cardiovascular diseases. The treatment of such chronic diseases requires proper management of hyperlipidemia in order to treat them. The current treatments available include mainly statins and its derivatives like fluvastatin, pravastatin, and rosuvastatin. Other agents including omega 3 fatty acids, ezetimibe, fibrates, Bile acid binding resins, HMG-CoA reductase inhibitors (statins), Nicotinic acid (niacin) Though these agents are popularly used for the treatment of hyperlipidemia, but also pose serious side effects on human health. Many investigators have reported the adverse side effects of putative therapeutic agents leading to health hazards in treated patients *viz.*, bile acid binding resins produce constipation, nausea, and bloating as side effects (Studer et al.,2005), ezetimibe is considered to be well tolerated as monotherapy, side effect profile similar to placebo (Morris et al., 2003), fibrates produce gastrointestinal upset, rash, and abdominal pain (Jun et al., 2009), nicotinic acid side effect show flushing is common; May increase uric acid and glucose levels (Brown et al ., 2001); Omega-3 fatty acids treatment eventually leads to Dyspepsia (Zhao et al.,2009).

Therefore there is a great demand for alternative treatments that avail strong clinical benefits with no side effects. Food and Diet based therapy holds to be the best alternative of interest to be assessed for their hyperlipidemia properties. Thus the proposed study shall be focused on evaluation of minor millets based therapy against hyperlipidemia condition in in vivo models and unmask the molecular mechanism of action of potential constituent in millets through cell culture based molecular biology approach. Thus the proposed research shall yield a potential millet diet for its application in treatment of hyperlipidemia, which can be eventually leading to scaling-up of diet production.

INTERNATIONAL STATUS: World health organization (WHO) has referred cardiovascular disease as the most important disorder associated with hyperlipidemia, posing the mortality rate of up to 17.9 million every year, accounting to 31% of all global deaths. Reports indicate that more than 100 million (around 53%) of adults are reported to have elevated low density lipoprotein in United States. While adults in America have total cholesterol levels more than 240mg/dL, thus projecting the risk of cardiovascular diseases, which emphasizes the direct association of hyperlipidemia with cardiovascular disease (Murphy et al., 2012; Mozaffarian et al., 2016). National Nutrition and Health Survey of Chinese population on hyperlipidemia prevalence during 2002, 2007, 2014 and 2017 showed increasing trend in the prevalence with 18.6%, 33.97%, 41.9% and 42.8% of adult population respectively. Thus China holds the burden of hyperlipidemia as a one of the major public health problems (Jinhua et al., 2018). The earliest report of WHO during 2008 indicates that around 54% of European population is suffering from hyperlipidemia posing the highest prevalence in the world. Whereas, the prevalence of hyperlipidemia in African population and South East Asian population is reported to be 22.6% and 29% respectively (WHO, 2008). Australian Health Survey 2011/12

has reported that around 5.6 million adults have high total cholesterol, posing the prevalence of hyperlipidemia in 33% of total Australian population (Australian Bureau of Statistics; Australian Health Survey 2011/12).

According to the report of National Health and Nutrition Survey carried out in Japan in the year 2015 on adults, the prevalence of lipid levels in both gender was found to be 11% in men and 19% in women respectively (Ministry of Health, Labour, and Welfare; 2015 Japan). WHO statistics during 2013 revealed the prevalence of hyperlipidemia in Indonesian adults was 33% in men and 38% in women. New Zealand Health survey carried out in the year 2015 and 2016 reported elevated levels of lipids in 11.5% adult population.This clearly indicates that the hyperlipidemia is one of the major health related issues throughout the world. Thus the global financial burden that the world experiences in management of such wide spread health issue commonly observed in general adult population affecting their lifestyle and productivity of manpower is a questionable and important medical problem to be addressed with priority.

Current status of drug regimen for hyperlipidemia:

Weng et al., (2010) reported on prevention of hyperlipidemia through statins (HMG-CoA reductase inhibitors) based treatment to patients with highest risk of developing chronic hyperlipidemia using validated prediction models. However, statins not experimentally proved to cure anti-hyperlipidemia (Weng et al ., 2010). According to the studies reported by Hooper et al., (2004) even though omega-3 fatty acids were previously considered to reduce chronic hyperlipidemia risk. It is unclear whether if dietary or supplemental omega-3 fatty acids reduce the risk of total mortality or cardiovascular events in persons at high risk of CVD or in the general population. However, there is lack of evidence supporting action of omega-3 fatty acid against hyperlipidemia. Other reports indicate other than synthetic pharmacological options for treating hyperlipideima, other alternative agents of food products like garlic are also known to reduce plasma cholesterol levels (Stevinson et al., 2014).

Although statin based therapy by using atorvastatin, rosuvastatin, fluvastatin, and pitavastatin has made considerable amount of progress in their usage in treating hyperlipidemia, there are several side effects reported that might pose chronic health issues. Even though statins have been confirmed to show safety and efficient profile, they have reportedly showed evidences of causing several adverse side effects, and the condition is known as statin intolerance (Weng et al ., 2010). Statins are known to negatively affect fatty acid oxidation, sarcolemal cholesterol, deplete isoprenoid, disturb calcium homeostasis and lipid metabolism, which cause autoimmunity, and increase cellular lipids leading to emergence of statin-induced adverse effects (Stone, 2004). Reports also indicate that continuous usage of statins for longer period of time was noticed to increase the risk of diabetes (Sattar et al., 2011). Another study also reported that hyperlipidemic patients treated with intensive stain therapy showed faster risk of developing diabetes then compared the ones treated with moderate statin therapy (Preiss et al., 2010). There are evidences that have reported to cause liver dysfunction by continuous usage of statin therapy eventually leading to liver failure (Russo et al., 2009).

NATIONAL STATUS:

Hyperlipidemia or hypercholesterolemia is one of the major health ailments that have profound effect of general population health, affecting their lifestyle and performance. Importantly these disorders are showing increasing trends in the Indian population. An estimate of Indian population with increasing trends of elevated levels of total cholesterol from 1980 to 2008 was reported (Farzadfar et al., 2011).

One of the major risk factors of hyperlipidemia is cardiovascular diseases, according to the global burden of disease study (2010) cardiovascular diseases constitutes of around 24.8% of deaths in India. Other than CVD, obesity is also one of the leading risk factor associated with hyperlipidemia, which results due to elevated levels of cholesterol, and thus resulting in increase in body mass index or BMI. In India, more than 135 million individuals were affected by obesity. According to the data reported by ICMR, INDIA prevalence of hyperlipidemia among 79% of adults in 4 different parts of India with highest rate of prevalence *viz.*, Chandigarh, Jharkhand, Tamilnadu, and Maharashtra (Joshi et al., 2014). Indian statistical data on hyperlipidemia shows that higher triglycriede levels and lower HDL levels in Indians and South Asians migrants then compared to cholesterol levels in UK and US (Enas, 2000).

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IMPORTANCE OF THE PROPOSED RESEARCH IN THE CONTEXT OF CURRENT STATUS:

According to disease control data survey, hyperlipidemia is ranked second in the 10 most chronic diseases in India (year). Hyperlipidemia is proved to strongly associate with many chronic diseases such as diabetes, obesity, cancer, and cardiovascular diseases. The treatment of such chronic diseases requires proper management of hyperlipidemia in order to treat them. The current treatments available include mainly statins and its derivatives like fluvastatin, pravastatin, and rosuvastatin. Other agents including omega 3 fatty acids, ezetimibe, fibrates, Bile acid binding resins, HMG-CoA reductase inhibitors (statins), Nicotinic acid (niacin) Though these agents are popularly used for the treatment of hyperlipidemia, but also pose serious side effects on human health. Many investigators have

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Therefore there is a great demand for alternative treatments that avail strong clinical benefits with no side effects. Food and Diet based therapy holds to be the best alternative of interest to be assessed for their hyperlipidemia properties. Thus the proposed study shall be focused on evaluation of minor millets based therapy against hyperlipidemia condition in in vivo models and unmask the molecular mechanism of action of potential constituent in millets through cell culture based molecular biology approach. Thus the proposed research shall yield a potential millet diet for its application in treatment of hyperlipidemia, which can be eventually leading to scaling-up of diet production.

METHODOLOGY:

Objective 1: *In vivo* and *in vitro* investigations on antihyperlipidemia property of 5 different types of minor millets.

Sample collection: According to the broad classification by Indian Institute of Millets, Hyderabad, millets are classified as major millets and minor millets. The current study is focusing on 5 different minor millets that include kodo millet, barnyard millet, foxtail millet, porso millet and little millet. All samples of the millets will be collected from different agriculture institutes across India *viz.*, GKVK Bangalore, Indian Institute of Millet research Hyderabad, University of Agricultural Sciences Dharwad, Tamil Nadu Agriculture University Coimbatore, Regional Agriculture Research Station Nandyal Andra Pradesh, Indian council of agriculture research Pune, Acharya N G Ranga Agricultural University, Andhra Pradesh and Regional Agricultural Research Station and from All India Coordinated Research Project on Small Millets, Collage of Agriculture, Rewa (M.P.). All samples will be grown in sophisticated fields with regular monitoring in the local fields of Vijayapura, for obtaining roots, shoots and sprouts for the study.

Processing of minor millets: It is important to process millets in order to achieve several goals like, easy digestibility, food safety, for increasing nutrition value and organoleptic properties. *Germination*: In order to get sprouts of millets, germination holds important role. Germination will be carried out in small, well designed pots consisting of clean and fertile soil. Sprouts thus obtained will be dried in autoclave at 50˚c to remove moisture content. The dried sprouts will be further powdered and used for the studies.

Different methods of processing techniques will be used that includes - dehulling, hydrothermal processing, milling and sieving.

In vitro lipid degradation assay using GCMS

Different types of extracts from 5 millets will be tested for their in-vitro action on modification in fatty acid profile of soybean oil, along with reference lipase (commercial porcine pancreatic lipase) which will also subjected to performance analysis for comparative purposes. Here crude soybean oils will be allowed to react with 5% of different millet extracts over the reaction medium volume. Crude oil- and reagent blanks will be maintained. The samples will be analyzed by gas chromatography with mass spectrometry (GC/MS) in order to check whether the fatty acid methyl esters (FAE) profile changed in the different treatments. The identification of fatty acid esters will be accomplished by comparing the retention time between samples and the fatty acid ethyl esters by comparing the mass spectrum using the NIST (Nist62.lib and Wiley139.lib) and WebBook databases (http://webbook.nist.gov/chemistry/).

In vivo investigation of antihyperlipidemic activity of millets in rat models:

a) Animals

Experiments on rats will be performed according to the OECD guidelines and National Institutes of Health guidelines. Animals shall be treated humanely and with regard for the alleviation of suffering. Male Wistar albino rats weighing 120-130 g will be considered for the investigation. All rats will be randomly assigned to experimental groups. Experimental design includes, 8 groups of male rats (n=6), will be monitored in cages, in air condition rooms maintained at 22˚c with 12 hours dark-light cycle with proper ab-libitum. During the course of experiment, adequate quantity of food and water will be provided. Experimental protocol will be carried out through the approval of Institutional animal ethical committee (IAEC).

b) Induction of hyperlipidemia

c) Acute toxicity studies and prediction of LD_{50}

Acute toxicity studies will be carried out by step-up and step down method using rats (reference).

d) Experimental Designing

Group 1- fed with normal diet, Group 2- negative control (only high fat diet HFD), Group 3- positive control (HFD + standard drug), Group 4 and subsequent groups will be test millet extracts along with HFD.

e) Anthropometric analysis

f) Biochemical assays of blood

g) Histopathological examinations

Objective 2: Unmasking the molecular mechanism of antihyperlipidemia action of potential millet through transcriptomics analysis.

Animals

The control group rats (n=6) will be fed with a standard laboratory pelleted diet and free access to water (6% calories from fat), Hyperlipidemia group rats (n=6) will be fed fat-rich hypercaloric commercial diet and free access to water (65% calories from fat), whereas the millet diet treated group (n=6) will be fed with millet diet along with fat-rich hypercaloric commercial diet. These feeding processes will be for a period of 90 days. Food will be offered ad libitum and food intake and body weight shall be measured daily. After the experimental feeding period, animals will be euthanized and epididymal white adipose tissue will be collected and frozen in liquid nitrogen, and stored at -80 °C. Certain biochemical assays including serum lipid profile, blood glucose, plasma insulin, and leptins shall be carried out using auto-analyzers or using kits.

Objective 3: Analysis of gene expression with therapeutic action of millet against hyperlipidemia through bioinformatics approach.

Based on the gene expression data of each gene generated from transcriptomics analysis will be used for inferring pathway representation. This approach will implicitly assume each gene as target for enrichment. By using gene set approach the known functionally related genes together will be calculated for their statistical significance. The gene sets that are known to be in a functional relationship will be deduced using signals from gene expression data.

Signaling/metabolic pathway will be analyzed by integrating the knowledge from biological databases, the scientific literature and intrinsic knowledge from domain experts and relating with gene expression data. Pathway generation will be done using commercial software such as protein lounge. Here the deduced pathway will possess the genes that are upregulated or downregulated during hyperlipidemia and observed to be normalized due to millet diet treatment as analyzed by transcriptomics study. Hence, such information is important to be incorporated in identifying the significantly enriched pathways associated with hyperlipidemia and prophylactic effects of millet diet treatment.

Year	Achievable Objective
Year 1	***Objective 1*** *In vivo* and *in vitro* investigations on antihyperlipidemia property of 5 different types of minor millets.
Year 2	***Objective 2*** Unmasking the molecular mechanism of antihyperlipidemia action of potential millet through transcriptomics analysis.
Year 3	***Objective 3*** Analysis of gene expression with therapeutic action of millet against hyperlipidemia through bioinformatics approach.

Table 1. Time Schedule of activities giving milestones through BAR diagram.

EXPECTED RESEARCH OUTCOME:

- This work gives insight of anti-hyperlipedemic property of minor millets that can be used for treating hyperlipidemia.
- Proposed study unmasks the molecular mechanism of how exactly minor millets can reduce lipid levels in blood.
- The Ph.D. will conclude which among the minor millets has potential to treat hyperlipidemia.
- Transcriptomics analysis reveals the genes involved in potential minor millet that is responsible for decrease in lipid levels

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